

# Mutations in the *GGCX* and *ABCC6* Genes in a Family with Pseudoxanthoma Elasticum-Like Phenotypes

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A characteristic feature of classic pseudoxanthoma elasticum (PXE), an autosomal recessive disorder caused by mutations in the *ABCC6* gene, is aberrant mineralization of connective tissues, particularly the elastic fibers. Here, we report a family with PXE-like cutaneous features in association with multiple coagulation factor deficiency, an autosomal recessive disorder associated with *GGCX* mutations. The proband and her sister, both with severe skin findings with extensive mineralization, were compound heterozygotes for missense mutations in the *GGCX* gene, which were shown to result in reduced  $\gamma$ -glutamyl carboxylase activity and in undercarboxylation of matrix gla protein. The proband's mother and aunt, also manifesting with PXE-like skin changes, were heterozygous carriers of a missense mutation (p.V255M) in *GGCX* and a null mutation (p.R1141X) in the *ABCC6* gene, suggesting digenic nature of their skin findings. Thus, reduced  $\gamma$ -glutamyl carboxylase activity in individuals either compound heterozygous for a missense mutation in *GGCX* or with haploinsufficiency in *GGCX* in combination with heterozygosity for *ABCC6* gene expression results in aberrant mineralization of skin leading to PXE-like phenotype. These findings expand the molecular basis of PXE-like phenotypes, and suggest a role for multiple genetic factors in pathologic tissue mineralization in general.

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## INTRODUCTION

Pseudoxanthoma elasticum (PXE; OMIM 264800, 177850) is an autosomal recessive multisystem disorder characterized by dystrophic mineralization of soft connective tissues, particularly the elastic fibers, in a number of organs, including the skin, the eyes, and the arterial blood vessels (Ringpfeil *et al.*, 2001; Neldner and Struk, 2002; Uitto and Ringpfeil, 2007; Li *et al.*, 2008). The primary cutaneous lesions are small, yellowish papules on the predilection sites at flexural areas, and these lesions progressively coalesce into larger plaques of inelastic, leathery skin with a yellowish hue. Histopathology of the affected skin shows accumulation of pleiomorphic elastotic material in upper and mid dermis,

which is mineralized as visualized by special histopathologic stains. The eye manifestations consist of angioid streaks, and bleeding from the retina can result in loss of visual acuity and, rarely, blindness. The cardiovascular manifestations result from mineralization of the arterial blood vessels, and include gastrointestinal bleeding, intermittent claudication, hypertension and, occasionally, early myocardial infarcts. Although the disease has considerable morbidity and mortality, the phenotypic spectrum is highly variable with both inter- and intra-familial heterogeneity. The precise incidence of this condition is undefined, but the estimates are in the range of 1 in 50,000–75,000.

Classic PXE is caused by mutations in the *ABCC6* gene, which encodes a putative transmembrane transporter protein, ABCC6 (also known as multidrug resistance-associated protein 6), a member of the family of ATP-binding cassette proteins (Bergen *et al.*, 2000; Le Saux *et al.*, 2000; Ringpfeil *et al.*, 2000; Miksch *et al.*, 2005; Pfendner *et al.*, 2007). The *ABCC6* gene is primarily expressed in the liver, to a lesser extent in the proximal tubules of kidneys, and at very low level, if at all, in tissues affected in PXE (Belinsky and Kruh, 1999; Scheffer *et al.*, 2002; Matsuzaki *et al.*, 2005). Although the mineral deposits in the affected tissues are known to consist of calcium and phosphate, the precise mechanisms leading to aberrant mineralization remain unclear, and specifically, the substrate specificity of ABCC6 *in vivo* is currently unknown.

Occasionally, PXE-like phenotypes have been reported in association with multiple coagulation factor deficiency (Rongioletti *et al.*, 1989; Le Corvaisier-Pieto *et al.*, 1996;

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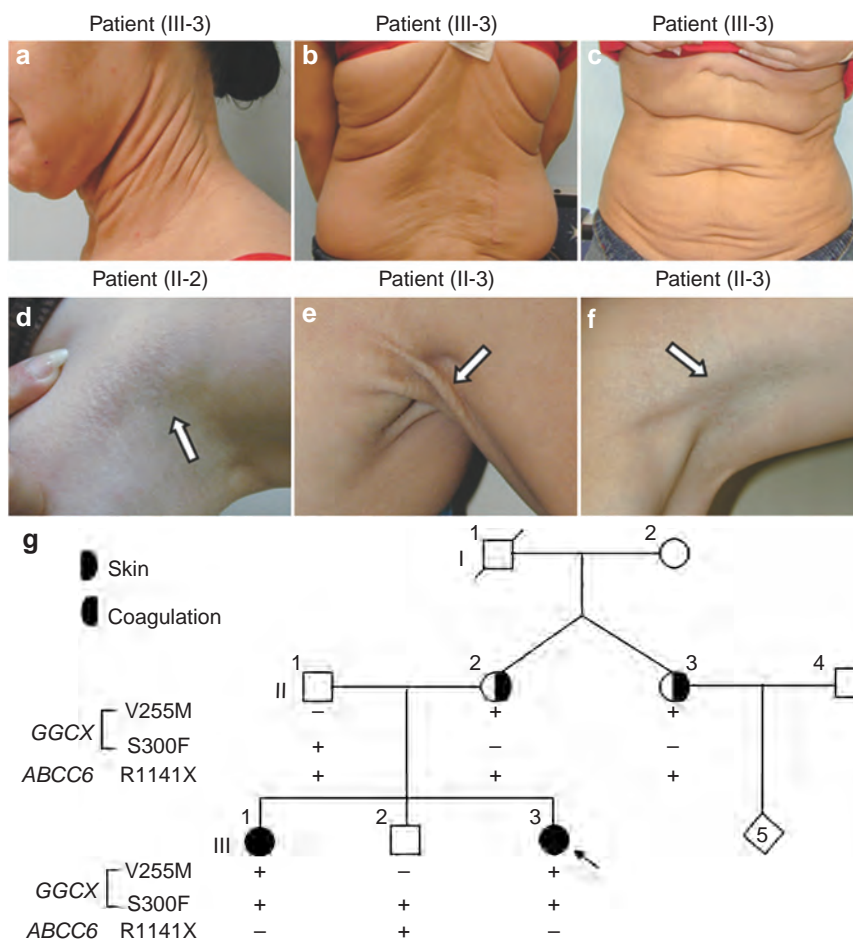
Vanakker *et al.*, 2007), and molecular analysis of some of these patients revealed mutations in the *GGCX* gene, which encodes an enzyme required for  $\gamma$ -glutamyl carboxylation of gla proteins (Vanakker *et al.*, 2007). In this report we detail a family with PXE-like clinical features, and also with deficiency of vitamin K-dependent clotting factors, particularly Factor X. Strikingly, this family harbors mutations both in the *GGCX* and *ABCC6* genes, and combinations of these mutations in two patients with PXE-like skin phenotype suggest digenic inheritance.

## RESULTS

### Clinical findings

The proband (III-3 in Figure 1) is a 16-year-old woman who was initially evaluated in early childhood because of cardiac abnormalities, including supraventricular pulmonic stenosis and peripheral pulmonary artery stenosis. She has undergone two pulmonary valve replacement procedures. Following an episode of endocarditis, she had a stroke with vision loss in the right eye, but rigorous ophthalmologic examination with

respect to angioid streaks has not been reported. She developed focal segmental glomerulosclerosis, thought to be immune complex mediated. At around age 10 years, she began developing progressively loose, sagging and redundant skin, primarily affecting the neck and trunk, and an initial diagnosis of cutis laxa was made (Figure 1a–c). She was also found to have a coagulation disorder with Factor X deficiency (Table 1). The proband has a 19-year-old sister (III-1) with similar skin changes beginning in her early teens, as well as a coagulation disorder; she has had a normal cardiac evaluation. They have a 17-year-old brother (III-2) who is clinically normal. Examination of other members of the nuclear pedigree (Figure 1g) showed that the father of the proband is clinically normal, whereas the mother (II-2), at the age of 40 years, has distinct folding of the skin, particularly in the axillary areas. She also has characteristic yellowish papules consistent with PXE on the sides of the neck and in the axillary areas (Figure 1d). The mother has a fraternal twin sister (II-3) with similar cutaneous findings (Figure 1e and f). Neither the mother nor her sister have evidence of a coagulation disorder (Table 1).



**Figure 1. Cutaneous findings in a family with PXE-like phenotype.** Note the loose and sagging skin in the 16-year-old proband (III-3, a–c). The proband's mother (II-2, d) and aunt (II-3; e, f), both 40 years of age, demonstrate redundant folding of the skin in the axillary area (d, e) and popliteal fossa (f), as well as small yellowish papules characteristic of PXE (arrows). The nuclear pedigree of the family with PXE-like skin findings and coagulation factor deficiency (g). The mutations in the *GGCX* and *ABCC6* genes identified in this family are indicated on the left of panel g. The proband is identified by an arrow.

Histopathology of the skin from the proband, her sister and the aunt revealed accumulation of basophilic elastotic material in the mid dermis, which was demonstrated to show aberrant mineralization with von Kossa and Alizarin red special stains (Figure 2). Thus, these patients have clinical and histopathological features of PXE.

### Mutation analyses

Considering the presence of PXE-like cutaneous findings, we first sequenced the *ABCC6* gene. The recurrent mutation,

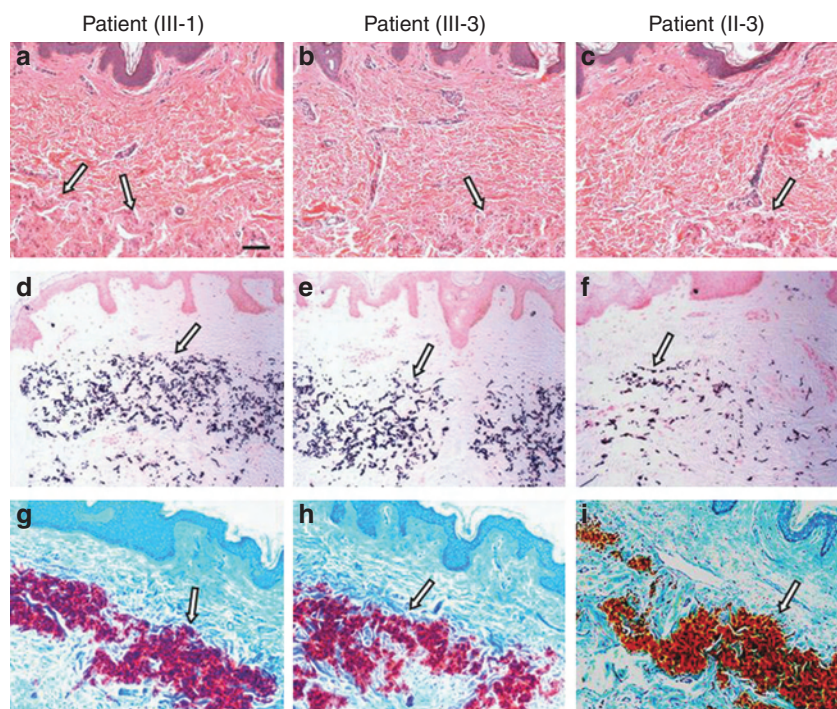
p.R1141X, which is particularly prevalent (about 30% of all detected mutations in PXE patients of the European extraction; Pfendner *et al.*, 2007) was found in the DNA from the father, mother, the maternal aunt and the younger brother, but not in the proband herself or her sister (Figures 1g and 3a). The individuals were heterozygotes for this mutation, and no other *ABCC6* mutation could be disclosed in this family. Sequencing of the proband's sister's and her aunt's *ABCC6* gene revealed the presence of eight heterozygous polymorphisms in the exons 7–19. This finding argues against large allelic deletions at the 5' half of the gene, which might not be detectable by the mutation detection strategy employed in our study (Pfendner *et al.*, 2007).

Considering the clinical association of the PXE-like cutaneous features with coagulation disorder in this family, we also sequenced the *GGCX* and *VKORC1* genes. The results demonstrated the presence of two missense mutations in *GGCX*. First, a single base transition mutation (c.791G→A) resulting in substitution of a valine by methionine at position 255 (p.V255M) of the  $\gamma$ -glutamyl carboxylase enzyme was detected (Figure 3b). This mutation was not present in 100 control alleles by restriction enzyme digestion and/or by direct nucleotide sequencing (Figure 3c). Secondly, a single nucleotide substitution (c.927C→T) resulting in substitution of a serine by phenylalanine in position 300 (p.S300F) was detected (Figure 3d). Direct sequencing of 150 control alleles detected the S300 codon only, suggesting that this missense substitution is not a frequent polymorphism. Determination of the evolutionary sequence conservation of *GGCX* in different species revealed that the amino acid corresponding to V255

**Table 1. Activities of the coagulation factors and clinical laboratory values in patients with *GGCX* gene mutations**

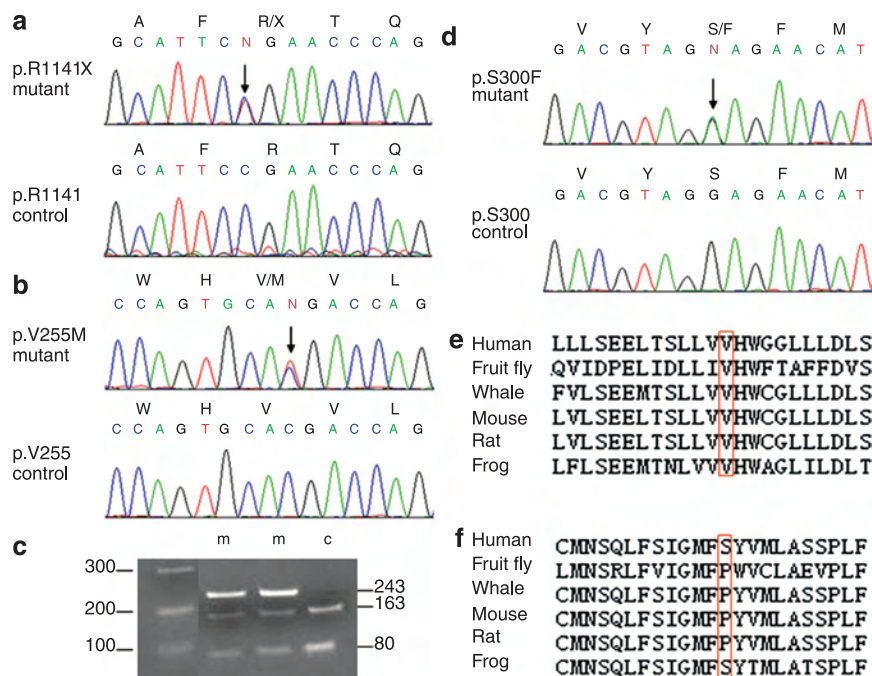
Laboratory measurement	Patient			Normal values
	III-1	III-3	II-3	
F II (%)	43	64	95	60–160
F VII (%)	31	108	86	60–160
F VIII (%)	–	67	–	60–160
F IX (%)	56	62	76	60–160
F X (%)	18	33	90	60–160
PTT (s)	32.1	43.3	32.0	25–41
PT (s)	21.6	18.1	10.9	12–15
INR	1.9	1.2	1.1	0.8–1.2

F, factor; INR, international normalized ratio; PT, prothrombin time; PTT, partial thrombin time.



**Figure 2. Histopathology of the cutaneous lesions in the sister (III-1), the proband (III-3) and aunt (II-3).** Staining with hematoxylin-eosin (a–c) demonstrates basophilic abnormal elastic structures in the mid dermis (arrows). Special stains (von Kossa, d–f; Alizarin red, g–i) reveal that these elastotic structures are mineralized (arrows). Scale bar = 0.1 mm.





**Figure 3. Mutation detection in the family.** (a) Identification of the recurrent nonsense mutation p.R1141X in the *ABCC6* gene. Note the heterozygous C→T transition substitution at nucleotide position 3,421 (arrow). (b, d) Identification of missense mutations p.V255M and p.S300F in the *GGCX* gene. The sequences shown are in the complementary strand. (c) The presence of the p.V255M mutation in the control population was examined by restriction enzyme digestion with *Apa*I. Note that the mutation abolishes the restriction site, resulting in the 243 bp mutant allele, in addition to 163 and 80 bp fragments. In control individuals the 243 bp PCR product is digested to 163 and 80 bp fragments (m, heterozygous mutant; c, control). (e, f) Evolutionary conservation of the amino acids V255 (e) and S300 (f) in the  $\gamma$ -glutamyl carboxylase protein.

is fully conserved (Figure 3e). In contrast, the serine at position 300 was less well conserved, several species depicting proline in this position, although the surrounding amino-acid sequences were highly conserved. For example, the sequence homology between the human and mouse 23-amino-acid sequence segment surrounding the S300 was 95.7% (Figure 3f). Sequencing of the *VKORC1* gene did not disclose any pathogenic mutations.

### Genotype/phenotype correlations

A correlation of the clinical findings with the genotypes revealed that the proband and her sister were compound heterozygotes for the two *GGCX* missense mutations, potentially explaining their hematologic findings (Brenner *et al.*, 1998; Spronk *et al.*, 2000; Rost *et al.*, 2004b, 2006). In contrast, the proband's father, brother, her mother, and the mother's twin sister were heterozygous for one of the *GGCX* mutations only, designating them as carriers without clinical hematologic findings (Figure 1g). The latter individuals were also carriers of the *ABCC6* nonsense mutation p.R1141X. Specifically, the mother and her twin sister were heterozygous for the *GGCX* missense mutation p.V255M and the *ABCC6* nonsense mutation p.R1141X, suggesting digenic inheritance of their cutaneous findings. However, the proband's younger brother and father were heterozygous carriers of the p.S300F mutation in the *GGCX* gene although they also carried the p.R1141X mutation in the *ABCC6* gene; they did not display any signs of cutaneous findings or hematologic disorder.

### Assay of $\gamma$ -glutamyl carboxylase activity

Previous studies have clearly demonstrated that the p.R1141X mutation in the *ABCC6* gene in heterozygous carriers does not cause PXE (Wegman *et al.*, 2005; Pfendner *et al.*, 2007). Similarly, combined coagulation factor deficiency is an autosomal recessive disease. The proband and her sister showed milder hematological defects than previously reported in patients with combined deficiency of vitamin K-dependent coagulation factors, which we postulated might be because of the type of missense mutations. We ascertained, therefore, a hypothesis that the missense mutations disclosed in the *GGCX* gene in this family may not result in complete loss of  $\gamma$ -carboxylase activity, therefore explaining the milder hematologic findings in these compound heterozygous individuals. Consequently, we measured the  $\gamma$ -glutamyl carboxylase activity of recombinant protein expressed from the wild-type (WT) as well as from mutant alleles harboring either the p.V255M or the p.S300F mutation.

The  $\gamma$ -glutamyl carboxylase activity was measured by using a small peptide containing the Phe-Leu-Glu-Glu-Leu sequence as substrate, in the absence of Factor X propeptide. The assay, which utilizes incorporation of radioactive [ $^{14}$ C]-CO<sub>2</sub> to the substrate, revealed clearly measurable activity with the WT enzyme (Table 2). In contrast, the mutant protein with the p.V255M mutation showed activity, which was only about 5% of that noted with the WT protein, and even more strikingly, essentially no activity (<1%) was detected with the protein harboring the p.S300F mutation (Table 2). To further examine the consequences of the

mutations on the  $\gamma$ -glutamyl carboxylase activity, additional measurements were performed with the same peptide substrate but also with a Factor X propeptide present in the reaction. This propeptide serves as the primary binding site for the carboxylase in Factor X and is conserved in vitamin K-dependent coagulation factors (Berkner, 2005). In the presence of the propeptide, the activity of the WT enzyme increased by about three-fold as compared to that in the absence of propeptide, whereas the protein with p.S300F

mutation continued to show low,  $\sim 3\%$  of the WT, activity (Table 2). In contrast, the activity of the protein harboring the p.V255M mutation showed little difference ( $\sim 90\%$ ) from the WT enzyme (Table 2). These observations clearly suggest that the mutations detected in the *GGCX* gene in this family are associated with reduced  $\gamma$ -carboxylase activity, but the reduced activity may result from different mechanisms with respect to enzyme-substrate interactions.

#### Reduced $\gamma$ -glutamyl carboxylation of matrix gla protein in the skin and plasma

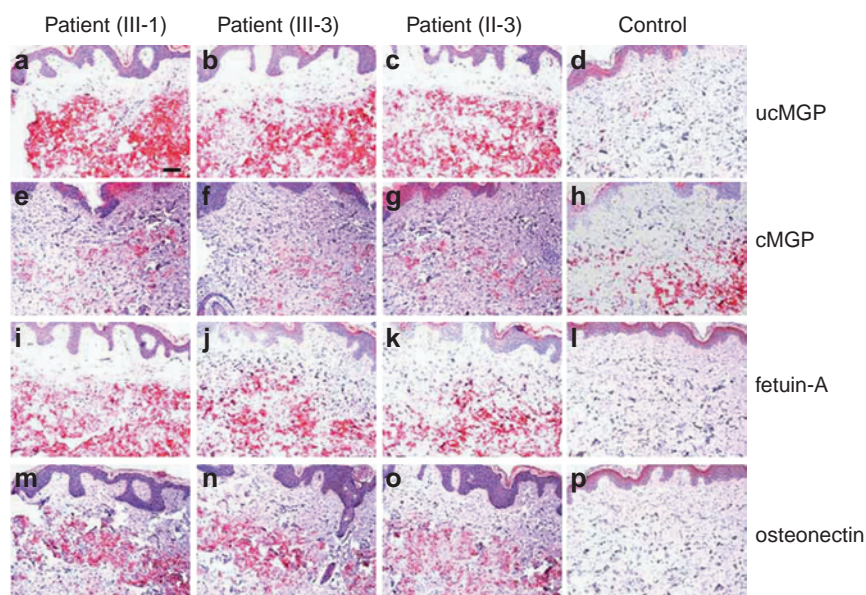
To examine the consequences of reduced  $\gamma$ -glutamyl carboxylase activity as a result of *GGCX* mutations, we next performed immunohistochemistry of skin biopsies with antibodies, which distinguish carboxylated and undercarboxylated forms of matrix gla protein (cMGP and ucMGP, respectively), a substrate for the carboxylase activity (Schurgers *et al.*, 2005). Staining of skin from the proband (III-3), her sister (III-1) or her aunt (II-3) revealed an abundance of MGP in the area of mineralization in the mid dermis, however, this protein was predominantly in the undercarboxylated form (ucMGP; Figure 4a–c). In contrast, specimens from normal unrelated control individuals revealed very little, if any, of ucMGP in the corresponding areas of dermis (Figure 4d). Low levels of cMGP both in patients' skin (Figure 4e–g) and in control skin (Figure 4h) were present. Thus, the ratio of uc/cMGP is markedly higher in the patients' skin, apparently reflecting reduced  $\gamma$ -glutamyl carboxylase activity. Immunostaining for two other proteins, fetuin-A and osteonectin, involved in the mineralization processes (Davies *et al.*, 2006; Jiang *et al.*, 2007), was also performed (Figure 4). The presence of both these proteins was noted in the patients' skin corresponding to the general areas of mineralization

**Table 2.  $\gamma$ -Glutamyl carboxylase activities of wild-type and mutant proteins *in vitro*<sup>1</sup>**

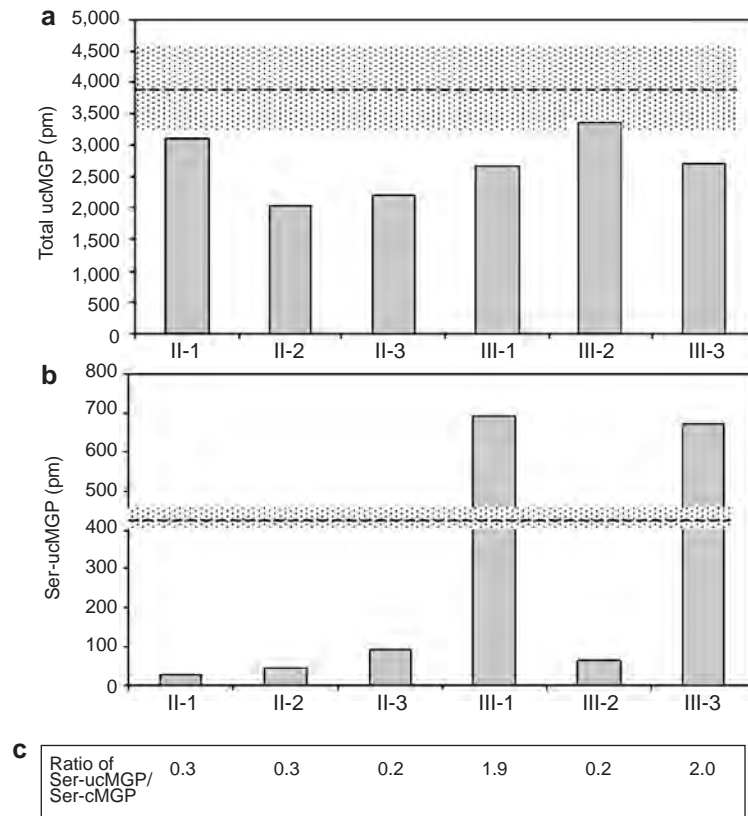
Variant	Carboxylase activity			
	Without propeptide		With propeptide	
	pmol h <sup>-1</sup>	Percent <sup>2</sup>	pmol h <sup>-1</sup>	Percent <sup>2</sup>
Wild type	3,649	100	10,379	100
S300F	28	<1	283	3
S300F	32	<1	301	3
V255M	211	5	9,427	91
V255M	156	4	8,994	87
Mock	16	0	19	0

<sup>1</sup>Solubilized microsomes from insect cells mock-infected or infected with baculovirus containing *GGCX* cDNA variants were assayed for expressed enzyme activity in the presence or absence of Factor X propeptide. Two independent viruses of each mutant were analyzed. The microsomes were also assayed by a quantitative western analysis, and the activities shown are normalized for equivalent amounts of carboxylase protein.

<sup>2</sup>The percent value was determined after subtracting the background observed in the mock-infected sample, the corresponding wild-type activity being 100%.



**Figure 4. Immunohistochemistry of skin in patients with PXE-like cutaneous lesions with mineralization (Patients III-1, III-3 and II-3) as well as in normal skin (control).** Monoclonal antibodies recognizing the undercarboxylated (ucMGP; a–d) and carboxylated (cMGP; e–h) forms of matrix gla protein, as well as polyclonal antibodies for fetuin-A (i–l) and osteonectin (m–p) were utilized. The secondary antibodies, biotin-conjugated anti-IgG, were recognized by avidin-alkaline phosphatase conjugates and visualized by incubation with an alkaline phosphatase substrate yielding red color. Scale bar = 0.1 mm.



**Figure 5. Quantitation of different forms of MGP in plasma by ELISA.** (a) Total, both phosphorylated and non-phosphorylated forms of undercarboxylated MGP (total ucMGP). (b) Assay of non-phosphorylated, undercarboxylated forms of MGP (ser-ucMGP). (c) The ratio of ser-ucMGP/ser-cMGP. The dashed lines in (a) and (b) represent the mean of control specimens and the coefficient of variation (CV) is indicated by the stippled areas (CV = 15.7% in a,  $n = 5$ ; CV = 6.0% in b,  $n = 100$ ).

(Figure 4i-k and m-o). In contrast, no staining was observed in normal skin (Figure 4l and p).

In addition to tissue analysis, we utilized a competitive ELISA to quantitatively determine the levels of total ucMGP in plasma, based on an antibody that recognizes both the phosphorylated and unphosphorylated forms of ucMGP. The results revealed that the father (II-1) and brother (III-2) of the proband in this family had values that were at the lower end of those in five unrelated controls (mean  $\pm$  standard deviation,  $3,902 \pm 612$  pM; Figure 5a). All the other members of the family showed reduced values, apparently reflecting deposition of ucMGP in the mineralized tissues. Assay of non-phosphorylated forms of ser-ucMGP, which display free serine residues recognized by a specific antibody, revealed that the proband (III-3) and her sister (III-1) had values far exceeding those of the control group ( $432.0$  pM;  $n = 100$ , CV 6%), whereas the values in plasma from other members of the family were low (Figure 5b). These findings, together with similar assays of ser-cMGP in the patients' plasma, showed a markedly increased ratio of ser-ucMGP/ser-cMGP in the proband and her sister (2.0 and 1.9, respectively), whereas the values of the rest of the family were within the normal range (mean 0.32; Figure 5c). Thus, the proband and her sister clearly show an abundance of undercarboxylated and non-phosphorylated species of MGP. As these forms of MGP

are inactive, unable to prevent aberrant mineralization, these findings explain the tissue mineralization shown in the skin by specific histopathologic stains (see above).

## DISCUSSION

In this study, we have detailed a family with PXE-like cutaneous features, in association with combined coagulation factor disorder. Specifically, the proband's mother and her maternal aunt presented with skin manifestations typical of PXE, such as yellowish papules and/or plaques with dot-like depressions characterized by mineralization of elastic fibers in the reticular and mid dermis as viewed by light microscopy. The proband herself and her sister were more severely affected and they have significant sagging of the skin with reduced elasticity and resilience and loss of recoil. Histopathology of the skin from the proband and her sister revealed distinct mineralization of elastotic structures in the mid dermis, as visualized with von Kossa and Alizarin red stains. These clinical and histopathologic findings in the skin are consistent with PXE (Ringpfeil *et al.*, 2001; Neldner and Struk, 2002; Uitto and Ringpfeil, 2007).

A second phenotypic feature in this family is deficiency of vitamin K-dependent coagulation factors, particularly Factor X, which are synthesized in the liver and depend on vitamin K for their function (Berkner, 2005). Combined deficiency of



the vitamin K-dependent factors (vitamin K-dependent coagulation factor deficiency, VKCFD) is an autosomal recessive disorder, caused either by mutations in the *GGCX* gene (type I: VKCFD1—OMIM no. 277450) on chromosome 2p12, or in the *VKORC1* gene (type II: VKCFD2—OMIM no. 607473) on chromosome 16p11.2. These genes encode a vitamin K-dependent carboxylase and vitamin K 2,3 epoxide reductase, respectively (Wu *et al.*, 1991; Li *et al.*, 2004; Rost *et al.*, 2004a,b). Both enzymes are essential for posttranslational  $\gamma$ -glutamyl carboxylation of clotting factors, enabling them to attach to the phospholipid bilayer of membranes as an essential prerequisite for blood coagulation (Zhang and Ginsburg, 2004).

To elucidate the molecular pathogenesis of these phenotypes in this family, we initially sequenced the *ABCC6* gene in consideration of the presence of PXE-like cutaneous findings. The recurrent mutation p.R1141X was found in the DNA from the father, mother, the maternal aunt and the younger brother, but not in the proband herself or her sister. In addition, no other *ABCC6* mutations could be disclosed in this family. Considering the clinical finding of coagulation disorder of the proband and her sister, we also sequenced the *GGCX* and *VKORC1* genes, in which mutations cause the type I and type II vitamin K-dependent coagulation factor deficiency disorders, respectively. Sequencing of the *GGCX* gene revealed two distinct missense mutations in this family, and specifically, mutations p.V255M in exon 7 and p.S300F in exon 8 of the *GGCX* gene were identified. The proband and her sister were compound heterozygotes for the two *GGCX* missense mutations, whereas the proband's father, brother, her mother and maternal aunt were heterozygous for one of the *GGCX* mutations only (Figure 1g). Sequencing of the whole coding region of *VKORC1* gene did not reveal any pathogenic mutation.

Considering the genotype/phenotype correlations, the proband and her sister were compound heterozygotes for both *GGCX* missense mutations, potentially explaining their hematologic findings. In addition, their skin findings resemble those recently reported by Vanakker *et al.* (2007), in which six patients were demonstrated to have both coagulation factor deficiency and PXE-like skin changes because of mutations in the *GGCX* gene. In contrast, the proband's father, brother, her mother and maternal aunt were heterozygous for one of the *GGCX* mutations only, designating them as carriers without clinical hematologic findings. The latter individuals were also carriers of the *ABCC6* nonsense mutation p.R1141X. It should be noted that the mother and her twin sister were heterozygous for one of the *GGCX* missense mutation p.V255M and one *ABCC6* nonsense mutation p.R1141X, suggesting digenic inheritance of their cutaneous findings. The occurrence of digenic inheritance, although rare, is well established (see for example, Gao *et al.*, 2007; Gropman and Adams, 2007; Jin *et al.*, 2008; Nozu *et al.*, 2008). The chance of a combination of mutations in the *ABCC6* and *GGCX* genes is difficult to calculate, because the precise carrier frequency of the mutations in these genes is not known. It should be noted, however, that in a cohort of >300 families with PXE analyzed in our laboratory for

mutations in the *ABCC6* gene, five individuals were heterozygous carriers of a mutation (p.R1141X) in one *ABCC6* allele only, yet none of them carried a mutation in the *GGCX* gene (unpublished). Thus, the combination of mutations reported in this study in two different genes, yet resulting in PXE-like phenotype, is rare. Finally, a recent study has suggested that heterozygosity for a single mutation in the *ABCC6* gene may closely mimic PXE, particularly displaying ophthalmologic findings (Martin *et al.*, 2008).

To investigate the functional consequences of the carboxylase mutants, WT and mutant proteins were expressed in insect cells, which do not contain endogenous carboxylase activity but synthesize active enzyme from exogenously introduced cDNA. We tested for carboxylase activity in an assay system that measures [ $^{14}$ C]-CO<sub>2</sub> incorporation into a Glu-containing small peptide substrate in the presence or absence of the Factor X propeptide. This propeptide regulates the activity of carboxylases by tethering the vitamin K-dependent proteins to the enzyme and by activating the carboxylase (Knobloch and Suttie, 1987; Berkner, 2005). In the absence of the propeptide, both mutant carboxylases showed markedly reduced enzyme activity, less than 5% of the WT activity. However, addition of the propeptide to the assay system restored the activity of the p.V255M mutant to the WT level, whereas the activity of the p.S300F remained low, ~3% of the WT. These *in vitro* findings clearly suggest that the mutant enzymes may be functionally deficient *in vivo*, but the mechanisms of inhibition may be distinct, and may involve differential propeptide as well as substrate binding.

Until now, 11 families comprising a total of 20 patients with PXE-like skin changes and/or vitamin K-dependent coagulation factor deficiency, with 16 distinct mutations in the *GGCX* gene, have been reported (Table 3). The functions of the residues that are mutated are for the most part unknown as only two mutants (p.L394R and p.W501S) have been previously characterized to sufficiently reveal function. Those analyses showed that Leu394 and Trp501 are part of the carboxylase and propeptide-binding sites, respectively (Lin *et al.*, 2002; Mutucumarana *et al.*, 2000, 2003). Both mutations therefore influence the affinity of  $\gamma$ -glutamyl carboxylase for the vitamin K-dependent protein substrate. To carry out the carboxylation, the enzyme must also bind three other co-substrates, which are vitamin K, CO<sub>2</sub> and O<sub>2</sub>, but functional residues that interact with these substrates are unknown (Berkner, 2005). Thus, the carboxylation reaction is complex and only a few functional residues have been identified. It will be of interest to determine the precise mechanisms by which the p.V255M and p.S300F mutants analyzed in the present study impair carboxylation. Nevertheless, these mutants are the first ones specific to PXE-like cutaneous phenotype to have been tested for *in vitro* carboxylase activity. The reduced activity in p.V255M and p.S300F indicate the importance of Val255 and Ser300 to carboxylation and can explain the coagulation disorder and PXE-like cutaneous phenotype detected in the proband and her sister.

Less apparent, and perhaps more complex, are the mechanisms leading to cutaneous PXE-like phenotypes in

**Table 3. Distinct mutations reported in the *GGCX* gene in patients with pseudoxanthoma elasticum-like skin findings and/or VKCFD**

Family (No) <sup>1</sup>	Age (years)	Mutations						Phenotype		References
		Allele 1		Exon	Allele 2		Exon	PXE-like	VKCFD	
I (4)	NB	p.L394R	c.1209T>G	9	p.L394R	c.1209T>G	9	NR	+	Brenner <i>et al.</i> (1998)
II (1)	NB	p.W501S	c.1530G>C	11	p.W501S	c.1530G>C	11	NR	+	Spronk <i>et al.</i> (2000)
III (1)	1	p.R485P	c.1454G>C	11	Loss of exon 3	Intron 2G>T	3	NR	+	Rost <i>et al.</i> (2004a, b)
IV (1)	38	p.R485P	c.1454G>C	11	p.H404P	c.1239A>C	9	NR	+	Rost <i>et al.</i> (2006)
V (1)	2	p.W157R	c.497T>C	4	p.T591K	c.1800C>A	13	NR	+	Darghouth <i>et al.</i> (2006)
VI (1)	46	p.W493S	c.1506G>C	11 <sup>2</sup>	p.W493S	c.1506G>C	11 <sup>2</sup>	+	+	Vanakker <i>et al.</i> (2007)
VII (1)	67	p.R476C	c.1454C>T	10	—	—	—	+	+	Vanakker <i>et al.</i> (2007)
VIII (1)	32	p.R476H	c.1455G>A	10	—	—	—	+	+	Vanakker <i>et al.</i> (2007)
IX (2)	46, 44	p.Q374X	c.1148C>T <sup>3</sup>	8	p.G537Y	c.1339G>T <sup>4</sup>	12	+	+	Vanakker <i>et al.</i> (2007)
X (1)	40	p.F299S	c.924T>C	8	p.G558R	c.1700G>A	12	+	+	Vanakker <i>et al.</i> (2007)
XI (2)	16, 19	p.V255M	c.791G>A	7	p.S300F	c.927C>T	8	+	+	Present study
XI (2)	40	p.V255M	c.791G>A	7	—	—	—	+	ND	Present study
XI (2)	17, 39	p.S300F	c.927C>T	8	—	—	—	ND	ND	Present study

NB, new born; ND, not detected; NR, not reported; PXE, pseudoxanthoma elasticum; VKCFD, vitamin K-dependent coagulation factor deficiency.

<sup>1</sup>The numbers in parenthesis indicate the number of individuals depicting the combination of mutations in the family.

<sup>2</sup>This mutation was reported to reside in exon 12 (Vanakker *et al.*, 2007).

<sup>3</sup>This mutation was reported as c.1149C>T (Vanakker *et al.*, 2007).

<sup>4</sup>In the reference sequence (NM000821), this nucleotide resides in exon 10 and encodes a tryptophan.

this family. Mutations in the *ABCC6* gene have been shown to cause PXE, an autosomal recessive disorder (Ringpfeil *et al.*, 2006), but heterozygous carriers of mutations, such as p.R1141X detected in the proband's mother and aunt, are asymptomatic (Wegman *et al.*, 2005; Pfendner *et al.*, 2007). At the same time, the proband and her sister did not harbor mutations in *ABCC6*, yet their skin lesions clearly showed profound mineralization. These findings raise the hypothesis that reduced carboxylase activity results in undercarboxylation of not only the coagulation factors but other proteins, such as MGP, as well. MGP plays a critical role in preventing aberrant mineralization but to be activated, this protein has to be fully carboxylated whereas undercarboxylated forms of the protein are inactive and can not prevent aberrant mineralization (Price *et al.*, 1998; Shearer, 2000). In fact, skin biopsies from the proband and her sister depicted preponderance of undercarboxylated MGP, when examined by immunohistochemistry using specific antibodies that distinguish the fully carboxylated and undercarboxylated forms of the protein (Schurgers *et al.*, 2005). Furthermore, assay of total ucMGP in plasma, which has been recently suggested to serve as a biomarker of cardiovascular calcification (Cranenburg *et al.*, 2008), was reduced in patients with skin findings, apparently reflecting tissue mineralization.

An intriguing observation in our family was the presence of PXE-like cutaneous features, with profound mineralization, in the proband's mother and aunt. These two individuals were heterozygous carriers of p.R1141X mutation in *ABCC6* and p.V255M in *GGCX*. As heterozygous carriers of p.R1141X in *ABCC6* alone do not manifest PXE and *GGCX*

mutations with respect to coagulation disorder are recessive, these findings suggest that the skin phenotype in these two individuals may be because of digenic inheritance. In this case, haploinsufficiency of the carboxylase activity and reduced *ABCC6* functions could be complementary or synergistic. The reasons for the fact that the proband's father and her brother were heterozygous carriers of mutations in the *ABCC6* gene (p.R1141X) and the *GGCX* gene (p.S300F) yet did not display any cutaneous findings are not clear. Specifically, whereas both *GGCX* mutations resulted in reduced enzyme activity, the reduction in case of protein harboring the p.S300F mutation was more pronounced than that of p.V255M. In this context, it should be noted that the substrate employed in the carboxylase assay is a pentapeptide, Phe-Leu-Glu-Glu-Leu, and it is possible that the activity measurements if carried out on full-length MGP as substrate would show differential activity with these two mutant enzymes. Finally, these two individuals have not been examined by an ophthalmologist nor has a skin biopsy been performed, because of their unwillingness to participate in further studies. Consequently, the possibility that the father and the brother of the proband may have subclinical, PXE-associated features can not be rigorously excluded.

The findings of this study have potential implications regarding the pathomechanisms of PXE, a multisystem disorder with extensive tissue mineralization. Our results suggest that undercarboxylation of MGP plays a critical role in aberrant mineralization of tissues in PXE, a proposition that has been supported by recent electron microscopic examination of skin of patients with PXE (Gheduzzi *et al.*, 2007) and analysis of the carboxylation status of MGP in *Abcc6*<sup>−/−</sup> null



mice, which recapitulate the features of PXE (Jiang *et al.*, 2007; Li *et al.*, 2007). How the absence of ABCC6, a putative transmembrane transporter expressed primarily in the liver, contributes to reduced  $\gamma$ -glutamyl carboxylase of MGP remains to be explored, but one possibility revolves around cellular transport and redistribution of vitamin K, an obligatory cofactor of  $\gamma$ -glutamyl carboxylase (Berkner, 2005; Schurgers *et al.*, 2007; Borst *et al.*, 2008; Li *et al.*, 2008). Such notion would have broader implications for aberrant tissue mineralization including pathologic processes, such as arteriosclerosis, beyond PXE. Finally, the ratio of ser-ucMGP/ser-cMGP was markedly elevated in the proband and her sister, individuals with most severe skin manifestations, including profound mineralization of the dermis. This ratio may reflect vitamin K status of the individual patient, and consequently, one could ascertain the possibility that treatment with appropriate vitamin K preparations could ameliorate or even reverse tissue mineralization in PXE, a currently intractable disorder.

## MATERIALS AND METHODS

### Patients

The patients were clinically evaluated at Washington University School of Medicine (St. Louis Children's Hospital and the Center for Advanced Medicine) and at St. Louis University. Informed consent was obtained from all patients. Skin biopsies were evaluated with light microscopy using hematoxylin-eosin, von Kossa and Alizarin red stains. Coagulation assays were performed on EDTA blood samples and the activities of Factors II, VII, VIII, IX, and X were measured in one-stage clotting assays.

### Molecular analysis

Genomic DNA was isolated from peripheral blood samples (QIAamp Blood Maxi kit; Qiagen Inc., Valencia, CA). PCR was performed using 1.5 U Taq polymerase (Qiagen) mixed with 5 U Optimase Taq polymerase (Transgenomic, Gaithersburg, MD) and Q buffer (Qiagen), according to the manufacturers' instructions. PCR reactions contained 200 ng DNA as template and 20 ng of each primer in a final volume of 50  $\mu$ l.

The *GGCX* gene was analyzed using primers and PCR conditions as previously described (Oldenburg *et al.*, 2000). The *ABCC6* sequences were analyzed using PCR primers as described previously (Pfendner *et al.*, 2007). For the detection of the deletion of exons 23–29, the primers described by Le Saux *et al.* (2001) were used. The *VKORC1* coding region was analyzed using primers described previously (Darghouth *et al.*, 2006).

The entire coding region and intron/exon boundaries of *GGCX*, *ABCC6* and *VKORC1* were analyzed with direct sequencing using an Applied Biosystems 3730 Sequencer (Applied Biosystems, Foster City, CA). Within the exon 7 of the *GGCX* gene, the 791G→A substitution abolished an *Apa*I restriction site. Digestion of the 243-bp PCR product resulted in the case of the normal allele in 163- and 80-bp fragments, whereas the heterozygotes showed 243-, 163- and 80-bp bands.

For *GGCX* sequences the nucleotide numbers are derived from cDNA (GenBank accession no. NM000821); the +1 corresponds to the –28 with respect to the translation initiation codon. In the *ABCC6* gene, the +1 correspond to the A nucleotide in the ATG

translation initiation codon (GenBank accession no. AF076622). The alignments of the *GGCX* protein sequences in different species were generated with Clustal W program.

### Construction and analysis of mutant carboxylase enzymes

Generation of carboxylase cDNAs with the individual mutations p.V255M and p.S300F was accomplished using the Quick Change XL site-directed mutagenesis kit (Stratagene, La Jolla, CA) according to the manufacturer's instructions and using the following oligonucleotide primers: p.V255M: (forward) CTGACTAGCCTGCTGGT CATGCACTGGGGTGGGCTGCTG and (reverse) CAGCAGCCCCA CCCCAGTGCATGACCAGCAGGCTAGTCAG; p.S300F: (forward) TTCAGCATTGGTATGTTCTTCTACGTCATGCTGGCCAGC and (reverse) GCTGGCCAGCATGACGTAGAAGAACATACCAATGCTGAA.

Briefly, a full-length *GGCX* cDNA was hybridized to the indicated primers and subjected to Pfu polymerase (Stratagene) extension. After 20 cycles of amplification, parental WT strands were digested with the methyl-sensitive restriction enzyme *Dpn*I, and amplified mutant strands were self-ligated and used to transform DH5 $\alpha$  *E. coli*. A *Bam*HI fragment containing the full-length cDNA was isolated and subcloned into the baculovirus expression vector pBacPak9 (Invitrogen Corp., Carlsbad, CA), and the cDNA was sequenced on both strands. Baculoviruses containing each variant were generated by cotransfecting SF21 cells with the plasmids and *Bsu*36I-digested viral DNA (BacPAK6; Clontech, Palo Alto, CA) as before (Rishavy *et al.*, 2004). Virus was isolated from individual plaques and screened for carboxylase expression by infecting cells, preparing lysates and performing Western analysis using an antibody against the C-terminus of the carboxylase (Berkner and McNally, 1997; Berkner and Pudota, 1998). Two independent viral isolates of each mutant were then amplified and used in large-scale infections to generate microsomes. The microsomes were solubilized (at 4 mg ml<sup>–1</sup>) in 0.5% CHAPS, 0.1% phosphatidyl choline and then assayed in a quantitative Western analysis to determine the amount of carboxylase protein. Carboxylase activity was measured by incubating the solubilized microsomes (40  $\mu$ g) in 160  $\mu$ l of 0.8 M ammonium sulfate, 0.16% CHAPS, 0.16% phosphatidyl choline, 2.5 mM Phe-Leu-Glu-Glu-Leu peptide as substrate, 50 mM BES (pH 6.9), 2.5 mM dithiothreitol, 1.3 mM [<sup>14</sup>C]-CO<sub>2</sub> and 200  $\mu$ M vitamin K hydroquinone. Samples were assayed in duplicate and the assays were performed either in the presence or absence of 10  $\mu$ M Factor X propeptide (Berkner, 2005).

### Immunohistochemistry

Immunohistochemistry was performed on tissues embedded in paraffin. Sections of 5  $\mu$ m were stained with moAb-cMGP (4.5  $\mu$ g ml<sup>–1</sup>; monoclonal antibody towards the carboxylated forms of human MGP), moAb-ucMGP (4.5  $\mu$ g ml<sup>–1</sup>; monoclonal antibody towards the non-carboxylated forms of human MGP) (Schurgers *et al.*, 2005), goat anti-human fetuin-A (1:40; R&D Systems, Minneapolis, MN) and rabbit anti-human osteonectin (1:1,000; Calbiochem, San Diego, CA), respectively. Immunostaining was performed using either biotinylated rabbit anti-mouse IgG (Dako, Carpinteria, CA) or biotinylated rabbit anti-goat IgG (Dako) or biotinylated swine anti-rabbit IgG (Dako) as secondary antibody (60 minutes at room temperature), followed by incubation with avidin-linked alkaline phosphatase complex (30 minutes at room temperature; Dako); staining was performed using the Vector

Red Alkaline Phosphatase Substrate kit I (Vector Laboratories, Burlingame, CA). Sections were counterstained with hematoxylin and mounted with Permount (Fisher, Fair Lawn, NJ). Controls for the immunoreactions were performed by omitting the primary antibody or by incubating the sections with nonimmune sera instead of the primary antibody.

### ELISA for plasma MGP

A conformation-specific monoclonal antibody (Schurgers *et al.*, 2005) was used to construct an ELISA for detection of total ucMGP in plasma, as described elsewhere (Cranenburg *et al.*, 2008). Two additional sandwich ELISAs were developed at VitaK BV (Maastricht, the Netherlands) to quantitatively measure the non-phosphorylated forms of both ucMGP and cMGP in plasma. In brief, a monoclonal antibody specific for non-phosphorylated-MGP (moAb serMGP) was coupled to the microtiter plate ( $5 \mu\text{g ml}^{-1}$ ). After incubation, non-specific sites were blocked using a solution of 2% BSA in Hepes/NaCl buffer. After stringent washing, standards, controls, or experimental samples were applied, diluted in 0.2% protifar in Hepes/NaCl buffer. The second antibody (either directed against the uncarboxylated or carboxylated conformation of MGP) was biotinylated and diluted in 0.2% protifar Hepes/NaCl buffer. After stringent washing, streptavidin in 0.5% BSA/Hepes/NaCl was added and staining was carried out using the substrate TMB (30 minutes at room temperature). The reaction was stopped with 2 N  $\text{H}_2\text{SO}_4$ , and the OD was measured at 450 nm.

We have received institutional approval of experiments and adhere to the Declaration of Helsinki Principles.

### CONFLICT OF INTEREST

The authors state no conflict of interest.

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